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(54) Title: TRANSGENIC ANIMAL MODEL FOR NEURODEGENERATIVE DISEASES

(57) Abstract: The present application refers to a mouse parkin2 DNA- and protein sequence, containing mutations or deletions causing Parkinson's disease in a human if occurring in the according human sequence, the construction of a transgenic non-human animal containing such a mutated DNA sequence and therefore expressing no or a less active or non-active parkin protein as well as the use of this transgenic animal as a model for neurodegenerative diseases.

Transgenic animal model for neurodegenerative diseases

The present invention relates to a mouse parkin2 DNA- and protein sequence containing naturally occurring or artificially introduced mutations or deletions, which cause Parkinson's disease in a human if they occur in the according human sequence, the construction of a truncated parkin gene, which expresses no, a non-active or a truncated parkin protein and a model of a transgenic animal, expressing such a less or non-active parkin protein instead of the native parkin protein or no parkin protein, as well as to the use of such a transgenic animal as a model for neurodegenerative diseases, preferred Parkinson's disease.

Neurodegenerative disorders are some of the most feared illnesses in society. During the last 10 years some of the genetic causes of many of the primary neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington´s disease, amyotrophic lateral sclerosis, prion disease and several ataxic syndromes, have been identified. These findings gave new insults in the knowledge about the initiating trigger as well as the resulting consequences of those diseases. Due to the fact that these diseases have many pathological mechanisms in common it seems possible that only relatively few pathways to neuronal death are involved in these disorders. Thus, treatment strategies for a particular neurodegenerative disease may be found to have value in other related disorders.

Parkinson's disease is a progressive neurodegenerative movement disorder with severe symptoms like rigidity, bradykinesia or tremor. The disease symptoms appear after degeneration of more than 70-80% of dopaminergic neurons. Broadly speaking the disease falls into two categories, namely late onset and early onset. Late onset, which occurs in older age (55+ years), mainly as consequence of environmental influences, leads to

enhanced dopaminergic neuron death at a faster rate and to a more severe degree than normal. Early onset Parkinson's disease is much more infrequent but starts between the ages of 35 and 60 years. There is evidence that three forms of this early type of Parkinson's disease show a tendency to run in families and is therefore known as familial Parkinson's disease.

In both the early and late onset types of Parkinson's disease, the pathology is the same but the abnormalities tend to be more severe and more widespread in cases beginning at an earlier age. The disease is characterised by lesions in brain areas where the cell bodies of the dopaminergic neurons are located mainly in the substantia nigra compacta. In addition intracytoplasmic inclusions known as Lewy bodies can be observed in different brain regions, in particular in substantia nigra and the locus ceruleus.

Recently two loci could be identified associated with early onset PD, one on human chromosome 4q21-23 ("PARK 1" gene locus) with a gene defect to be due to a missense mutation in the α-synuclein protein (or *parkin1*), a small abundant brain molecule (Polymeropoulos, M. *et al.*, Science 1997; 276:2045-2047), and one on chromosome 2p13 ("PARK 3" gene locus)(Gasser, T. *et al.*, Nat. Genet. 1998; 18: 262-265). Both forms are inherited in an autosomal dominant manner.

Lately an autosomal recessive form of familial Parkinson's disease could be observed, linked to human chromosome 6q25.2-27 ("PARK 2" gene locus) (Matsumine, H. *et al.*, Am J Hum Genet (1997); 60: 588-596). This gene, designated *parkin* (or later *parkin2*) contains 12 exons spanning more than 500 kb and encodes a protein of 465 amino acids (molecular weight 51,652 Dalton) with homology to ubiquitin at the N-terminal portion and a RING-finger like motif at the C-terminal portion.

It has been shown, that mutations in the α-synuclein gene lead to autosomal dominant Parkinson's disease (Polymeropoulos, M.. *et al.*, Science 1997; 276: 2045-2047), as well as mutations in the parkin gene cause autosomal recessive juvenile parkinsonism (Kitada, T. *et al.*, Nature 1998; 392: 605.608; Hattori, N. *et al.*, Biochem Biophys Res Comm 1998; 249: 754-758)).

Further Hattori, N. *et al.*, have been shown in Ann Neurol 1998; 44: 935-941, that different deletions in the parkin gene are the reason for truncated parkin proteins, causing autosomal recessive juvenile parkinsonism. Especially intragenic deletional mutations, involving exons 3 to 4, exon 3, exon 4 and exon 5, as well as exon 3 through exon 7 are described as effecting the disease. Deletion of exon 3 of the parkin gene is furthermore described by Lücking, C. *et al.* in the Lancet 1998; 352: 1355-1356 to cause autosomal recessive juvenile parkinsonism. Investigations of Abbas, N. *et al.* Human Molecular Genetics 1999; 8: 567-574 and Kitada, T. *et al.*, Nature 1998; 392: 805-808 show that mutations in the ubiquitin-like N-terminal part (exon 2) of the parkin gene can also cause autosomal recessive juvenile parkinsonism, as well as different frameshift- or missense mutations.

Leroy, E. *et al.*, demonstrated in Hum Genet 1998; 103: 424-427 that deletions of exons 5, 6 and 7 of the human parkin gene leads to early onset Parkinson's disease.

At present most common therapies are dealing with the increase of dopamine content in PD patients via application of L-dopa as precursor of dopamine, dopamine agonists or MAO-B (Monoamino Oxidase B) inhibitors, e.g. Deprenyl, by blocking the degradation of dopamine. There are no prophylactic therapies available to stop the progression of the degenerative disease before onset of symptoms in late onset PD. This is due to the fact that at present diagnosis is only possible when first symptoms occur. So

far it is not clear to which extent genetic components enhance the environmental components responsible for the increased cell death of dopaminergic neurons.

Although different transgenic animal models for neurodegenerative diseases like Alzheimer's disease have been created, a transgenic animal model for Parkinson's disease has not yet been described.

Homologous recombination may be employed for inactivation or alteration of genes in a site-directed manner. A number of papers describe the use of homologous recombination in mammalian cells, including human cells. Illustrative of these papers are Kucherlpati *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:3153-3157; Kucherlapati *et al.* (1985) Mol. Cell. Bio. 5:714-720; Smithies *et al.* (1985) Nature 317:230-234; Wake *et al.* (1985) Mol. Cell. Bio. 8:2080-2089; Ayares *et al.* (1985) Genetics 111:375-388; Ayares *et al.* (1986) Mol. Cell. Bio. 7:1656-1662; Song *et al.* (1987) Proc. Natl. Acad. Sci. USA 84:6820-6824; Thomas *et al.* (1986) Cell 44:419-428; Thomas and Capecchi (1987) Cell 51:503-512; Nandi *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:3845-3849; and Mansour *et al.* (1988) Nature 336:348-352. Various aspects of using homologous recombination to create specific genetic mutations in embryonic stem cells and to transfer these mutations to the germline have been described (Evans and Kaufman (1981) Nature 294:154-146; Dotschman *et al.*, (1987) Nature 330:576-578; Thomas and Capecchi (1987) Cell 51:503-512; Thompson *et al.* (1989) Cell 56:316-321. The combination of a mutant polyoma enhancer and a thymidine kinase promoter to drive the neomycin gene has been shown to be active in both embryonic stem cells and EC cells by Thomas and Capecchi, supra, 1987; Nicholas and Berg (1983) in Teratocarcinoma Stem Cell, eds. Siver, martin and Strikland (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (pp. 469-497); and Linney and Donerly, Cell 35:693-699, 1983.

The object of the present application is to provide the suppositions for a test model for neurodegenerative diseases, preferably Parkinson's disease and a valuable tool in the diagnosis and treatment of these conditions, as well as the development of experimental models of Parkinson's disease that can be used to define further the underlying biochemical events involved in the pathogenesis of this disease.

This object is met by a polynucleotide sequence encoding a mouse parkin2 protein, containing naturally occurring or artificially introduced mutations or deletions, which cause Parkinson's disease in a human if they occur in the according human sequence, a vector, containing such a sequence, a prokaryotic or eukaryotic cell, containing such a vector and a transgenic non-human animal, whose one or both alleles of a gene encoding a parkin gene are mutated in a way, that a protein with modified, preferred less activity or no active protein is expressed.

The transgenic non-human animals according to the present invention can be used as models for analysing the symptoms of neurodegenerative diseases or as a model system for testing the efficacy of a treatment for a neuro-degenerative disease, whereby it is not an object of the present application to provide any method for treating one of the described diseases in a human or animal.

Such models could presumably be employed, in one application, to screen for agents that alter the degenerative course of Parkinson's disease. For example, a model system of Parkinson's disease could be used to screen for environmental factors that induce or accelerate the pathogenesis. Further an experimental model could be used to screen for agents that inhibit, prevent, or reverse the progression of Parkinson's disease. Presumably, such models could be employed to develop pharmaceuticals that are effective in preventing, arresting, or reversing Parkinson's disease. Further such models can be used for examination of behaviour during the

development of a neurodegenerative disease, for examination of physiological and molecular biological correlation of the disease, for studies of drug effects and for determination of effective drug doses and toxicity. These applications should be considered as examples and should not limit the application of the models in any way.

The present invention provides model systems of neurodegenerative diseases, preferred Parkinson's disease, wherein the model system comprises a mutated isoform or a fragment of the mouse parkin2 gene (further designated as *mPark2*), a DNA sequence derived from SEQ ID NO: 1 encoding a mouse parkin2 protein corresponding to the human parkin protein encoded by human chromosome gene region 6q25.2-27 ("PARK 2" gene locus). Preferred the model system contains a mutated mPark2 sequence or a mPark2 sequence containing any deletion, coding for a mutated or truncated, less active or non-active parkin protein.

The sequence of human α-synuclein (*parkin1*) gene, as well as human parkin (*parkin2*) gene is known. Human parkin2 gene (further designated as *hPark2*) contains 12 exons, coding for a protein which has in full length 465 amino acids and a molecular weight of 51,652 Daltons.

The present application shows the full length cDNA of mPark2 in SEQ ID NO:1, consisting of 12 exons, containing the full length open reading frame for the mouse parkin2 protein (SEQ ID NO:4) which coding region consists of 1395 bp, coding for a protein of 464 amino acids with a calculated molecular weight of 51615 Dalton. Further two shorter cDNAs spanning a coding region of 789 bp (SEQ ID NO: 2 (isolated from mouse brain cDNA library by specific PCR)) and 753 bp (SEQ ID NO: 3 (isolated from mouse kidney cDNA library by specific PCR)) corresponding to amino acid sequences of 262 amino acids (SEQ ID NO:5) and 250 amino acids (SEQ ID NO:6) respectively are provided.

During the work of isolation and sequencing of the sequences SEQ ID NO: 1 to 3 shown in this application Shimizu, N. *et al.* submitted a mouse parkin DNA sequence to the EMBL GenBank database, published in July 1999 with the accession number AB019558. The protein sequence of the mouse parkin protein encoded by the published sequence is identical to SEQ ID No: 4.

The present invention refers to polynucleic acid sequences derived from SEQ ID NO: 1, containing naturally occurring or artificially introduced mutations or deletions, which are known to cause Parkinson's disease in a human if they occur in the according human sequence.

The present invention encompasses further polynucleotide sequences containing naturally occurring mutations according to the wobble principle, which represents the degeneration of the genetical code, as well as according to the polymorphism of the genetical code, encoding any protein which has the same or a homologous amino acid sequence as any of the mutated or truncated mouse parkin2 proteins of the present invention.

"Homologous amino acid sequence" in content with the mouse parkin2 protein means in the present application an amino acid sequence, wherein at least 70 %, preferably 80 %, more preferably 90 % of the amino acids are identical to one of the proteins of the present invention and wherein the replaced amino acids preferably are replaced by homologous amino acids. As "homologous" amino acids are designated which have similar features concerning hydrophobicity, charge, steric features etc. Most preferred are amino acid sequences, containing the species-dependent differences of the mouse amino acid sequence compared to human parkin protein shown in the alignment Figure No. 1. The alignment of the corresponding polynucleotide sequences with the exon boundaries is shown in Figure No. 2.

In the whole application for nucleotides and amino acids the usual designations (one-letter ore three-letter code) are used, known by any person skilled in the art.

The full length polynucleotide sequence of SEQ ID NO:1 or fragments thereof can be obtained by isolation of genomic DNA, containing exons and introns of the mPark2 gene, by RNA transcripts of the DNA or by the preparation of cDNA, containing only the exons of the mPark2 gene. Further the full length sequence as well as fragments thereof may be obtained by synthetical polymerisation of nucleotides.

A preferred polynucleotide sequence of the present application is a polynucleotide sequence derived from SEQ ID NO: 1, which is either mutated or in which parts of the sequence are deleted. Mutations, insertions or deletions may be located 5'upstream of the open reading frame (i.e. in the promotor-region), or they can concern one or more exons of the open reading frame. More preferred is a sequence, containing either a mutated full length sequence or fragments of SEQ ID NO:1, encoding a truncated parkin2 protein (i.e. by mutations leading to a STOP codon or by deletions) or no protein (i.e. if the mutation or deletion is located in the promoter-region in exon 1).

More preferred mutations or deletions concern either exon 1, wherein the promotor region is contained, or exon 3 and/or one or more of the other exons.

Most preferred the polynucleotide sequence of the present application is selected from SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17; SEQ ID NO: 18, SEQ ID NO:19 or SEQ ID NO:20 (see also Table 1 and 2).

One of the polynucleotide sequences SEQ ID NO:1 to 3 may be treated *in vitro* or *in vivo* by random or site-directed mutagenesis, by random or site-directed digestion, by recombination or fusion or any other method known of persons skilled in the art to obtain sequences derived from SEQ ID NO:1 containing mutations or deletions leading to a less active or to no parkin protein. Of course a person skilled in the art will understand that the present invention encompasses as well any construction in which parts of or the whole polynucleotide sequence encoding the parkin gene is deleted or replaced by another sequence (i.e. by a sequence encoding an antibioticum-resistance).

To obtain at least a transgenic non-human animal as a model for neurodegenerative diseases, the natural occurring sequence of the parkin gene in this animal may be replaced on one or both alleles of the chromosomes by a sequence of mPark2, containing mutations or deletions according to the present invention. These animals produce either less or less active or no parkin protein.

The transgenic animals of the present invention are created using targeted gene replacement, a sequence by which a specific DNA sequence of interest (target DNA) is replaced by an altered DNA (replacement DNA). The genome of embryonic stem (ES) cells is modified using homologous recombination (Capecchi, Science 1989; 244:1288 and U.S. Pat. No. 5,487,992). The embryonic stem cells are injected in blastocysts as an early state of the developing embryo. The blastocysts are then placed in a pseudopregnant female animal.

Briefly, a vector is constructed that carries the replacement DNA. Both ends of the replacement DNA are flanked by long DNA sequences homologous to the sequences flanking the target DNA. When the vector is introduced into ES cells, the homologous sequences align and recombination may take place. This results in the target DNA being exchanged for the replacement

DNA. The vector is not replicated in the cells and will be lost. The frequency of homologous recombination is low; thus, a screening system is used. The replacement DNA will contain a positive marker sequence, usually a neomycin resistance gene. Thus, any cells that incorporate the replacement DNA by homologous recombination will resist neomycin. By growing cells in medium containing the drug neomycin one can select only those cells containing the replacement DNA. The ES cells containing the replacement DNA are then inserted into recipient mouse blastocysts to create chimeric mice. Chimeras with germ cells derived for the altered ES cells transmit the modified genome to their offspring, yielding mice heterozygous for the target DNA (contain one target DNA and one replacement DNA). The heterozygotes are then bred with each other either to create mice homozygous for the replacement DNA and deficient in the target DNA or to maintain transgenic heterozygotes if the homozygotic mice are not viable.

The DNA will comprise at least a portion of the gene(s) at the particular locus with introduction of a lesion into at least one, usually both copies, of the native gene(s), so as to prevent expression of a functional parkin protein. The lesion may be an insertion, deletion, replacement or combination thereof. When the lesion is introduced into only one copy of the gene being inactivated, the (heterozygote) cells having a single unmutated copy of the target gene are amplified and may be subjected to a second transformation, where the lesion may be the same or different from the first lesion, usually different, and where a deletion, or replacement is involved, may be overlapping at least a portion of the lesion originally introduced. The resulting transformants are screened for the absence of the functional protein of interest and the DNA of the cell may be further screened to ensure the absence of a wild-type target gene. Alternatively, homozygosity as to a phenotype may be achieved by breeding hosts heterozygous for the mutation.

For the construction of a transgenic animal model according to the present application any suitable animal may be employed, however mammals are preferred. More preferred are rodents and most preferred are rats and mice.

In the following the single steps of creating the animal models will be described in detail.

Starting from a polynucleotide sequence encoding a parkin gene, preferably from a sequence encoding a mPark2 gene, more preferably from a sequence according to any of SEQ ID NO:1 to 3, most preferred from SEQ ID NO: 1 a desired mutation, insertion or deletion is introduced to the sequence. Methods to create mutations by random or site-directed mutagenesis or desired insertions or deletions by random or site-directed digestion and/or replacement are commonly known to persons skilled in the art and broadly described in the literature. The method how a mutation, insertion or deletion is introduced in the sequence is not relevant, however falls under the scope of the present invention, as long as any of the later described nucleotides, amino acids or sequences are involved.

The constructs may be modified to include functional entities other than the mutated sequence which may find use in the preparation of the construct, amplification, transformation of the host cell, and integration of the construct into the host cell.

The homologous sequence for targeting the construct may have one or more deletions, insertions, substitutions or combinations thereof. For example, the mPark2 gene may include a deletion at one site and an insertion at another site which includes a gene which may be used for selection, where the presence of the inserted gene will result in a defective inactive protein product. Preferably, substitutions are employed. For an inserted gene, of particular interest is a gene which provides a marker, e.g.,

antibiotic resistance such as neomycin resistance, including G418 resistance.

The deletion will be at least about 50 bp, or more usually at least about 100 bp, and generally not more than about 20 kbp, where the deletion will normally include at least a portion of the coding region including a portion of or one or more exons, a portion of one or more introns, and may or may not include a portion of the flanking non-coding regions, particularly the 5'-non-coding region (transcriptional regulatory region). Thus, the homologous region may extend beyond the coding region into the 5'-non-coding region or alternatively into the 3'-non-coding region. Insertions will generally not exceed 10 kbp, usually not exceed 5 kbp, generally being at least 50 bp, more usually at least 200 bp.

The homologous sequence should include at least about 100 bp, preferably at least about 150 bp, more preferably at least about 300 bp of the target sequence and generally not exceeding 20 kbp, usually not exceeding 10 kbp, preferably less than about a total of 5 kbp, usually having at least about 50 bp on opposite sides of the insertion and/or the deletion in order to provide for double crossover recombination.

Upstream and/or downstream from the target gene construct may be a gene which provides a tool to select out primary random integration of the construct in the genome. For this purpose, the herpes simplex virus thymidine kinase gene may be employed, since the presence of the thymidine kinase gene may be detected by the use of nucleoside analogs, such as Gancyclovir or Acyclovir, for their cytotoxic effects on cells that contain a functional HSV-tk gene. The absence of sensitivity to these nucleoside analogs indicates that homologous recombination has occurred.

The presence of the marker gene inserted into the gene of interest establishes the integration of the target construct into the host genome.

However, DNA analysis might be required in order to establish whether homologous or non-homologous recombination occurred. This can be determined by employing probes for the insert and then sequencing the 5' and 3' regions flanking the insert for the presence of the gene of interest extending beyond the flanking regions of the construct or identifying the presence of a deletion, when such deletion is introduced.

The polymerase chain reaction (PCR) may be used, with advantage in detecting the presence of homologous recombination (Kim and Smithies, (1988) Nucleic Acid Res. 16:8887-8903; and Joyner et al (1989) Nature 338:153-156). Primers may be used which are complementary to a sequence within the construct, usually complementary to the selection marker gene, and complementary to a sequence outside the construct and at the target locus. In this way, one can only obtain DNA duplexes having both of the primers present in the complementary chains in homologous recombination has occurred. By demonstrating the presence of the primer sequences or the expected size sequence, the occurrence of homologous recombination is supported. Any person skilled in the art knows how to determine the suitable PCR primers and conditions.

The construct may further include a replication system which is functional in the mammalian host cell. For the most part, these replication systems will involve viral replication systems, such as Simian Virus 40, Epstein-Barr virus, papilloma virus, adenovirus and the like.

Where a marker gene is involved, as an insert, and/or flanking gene, depending upon the nature of the gene, it may have the wild-type transcriptional regulatory regions, particularly the transcriptional initiation regulatory region or a different transcriptional initiation region. Whenever a gene is from a host where the transcriptional initiation region is not recognized by the transcriptional machinery of the mammalian host cell, a different transcriptional initiation region

will be required. This region may be constitutive or inducible, preferably inducible. A wide variety of transcriptional initiation regions have been isolated and used with different genes. Of particular interest as promoters are the promoters of metallothionein-I and II from a mammalian host, thymidine kinase, beta-actin, immunoglobulin promoter, human cytomegalovirus promoters, and SV40 promoters. In addition to the promoter, the wild-type enhancer may be present or an enhancer from a different gene may be joined to the promoter region.

The construct may further include a replication system for prokaryotes, particularly E. coli, for use in preparing the construct, cloning after each manipulation, allowing for analysis, such as restriction mapping or sequencing, followed by expansion of a clone and isolation of the plasmid for further manipulation. When necessary, a different marker may be employed for detecting bacterial transformants.

Once the vector has been prepared, it may be further manipulated by deletion of the bacterial sequences as well as linearisation, where a short deletion may be provided in the homologous sequence, generally not exceeding about 500 bp, generally being from about 50 to 300 bp. The small deletion will generally be near one or other end of the targeted structural gene.

The construction of the desired polynucleotide sequence may be carried out in a cloning vector and linearised prior to the transfection of ES cells. A broad range of cloning vectors as well as vectors for the homologous recombination are commercially available and may be selected according to the desired construction.

Cloning vectors are usually replicated in prokaryotic cells, which renders the selection and multiplication of the desired construct. It is not

critical which prokaryotic organism is used, but usually E.coli or a yeast strain is preferred.

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

Homologous recombination may be used to insert a mutant sequence into a host genome at a specific site, for example, at a host parkin locus. In one type of homologous recombination, one or more host sequence(s) are replaced; for example, a host parkin allele (or portion thereof) is replaced with a mutant parkin allele (or portion thereof). In addition to such gene replacement methods, homologous recombination may be used to target a mutant parkin allele to a specific site other than a host parkin locus. Homologous recombination may be used to produce transgenic non-human animals and/or cells that incorporate mutant parkin alleles.

Further to the above described techniques a step of expressing the treated sequence may be inserted in the expiration. Therefore the construct is (sub)cloned into any expression vector, which may be brought into a suitable eukaryotic cell. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired DNA sequences. Polynucleotides encoding a variant parkin2 polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences, such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art and is described further in Maniatis et al. Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor, N.Y. For example, but not for limitation, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

Any suitable eukaryotic cell may be used, but insect cells or mammalian cells as primary cells or immortalized cell lines are preferred.

A number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Baculovirus expression systems are useful for high level expression of heterologous genes in eukaryotic cells. Knops *et al.* (1991) J. Biol. Chem. 266(11):7285. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al. (1986) Immunol. Rev. 89:49, and necessary

processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, and the like. The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, microinjection of DNA into the nucleus or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al. Molecular Cloning: A Laboratory Manual, 2nd. Ed. Cold Spring Harbor Press, (1989). The DNA may be single or double stranded, linear or circular, relaxed or supercoiled DNA. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology (1990) 185:527-537.

For the creation of an animal model according to the present invention each polynucleotide sequence can be used, containing mutations, insertions or deletions which are known to cause Parkinson's disease in a human, when they occur in the corresponding human sequence. Preferred polynucleotide sequences for the creation of an animal model according to the present invention are those which mutations are shown in table 2. More preferred are polynucleotide sequences containing mutations or deletions shown in table 1. The most preferred polynucleotide sequence for the construction of a transgenic animal of the present invention is SEQ ID NO: 7
Further enclosed to the present invention is an animal model wherein the parkin sequence is replaced by an according sequence of another mammal (i.e. by the human sequence, containing one of the mutations, insertions or deletions described in the present application) or by a sequence encoding a marker, i.e. an antibioticum.

Table 1: Mutations or deletions in mPark2 cDNA (SEQ ID NO:1)

Position in SEQ ID NO:1	Replacement (DNA)	Replacement (protein)	SEQ ID NO (DNA seq)	SEQ ID NO (prot seq)
NT 300-540	Exon3	Frameshift, Truncation	7	21
NT 300-659	Exon3-4	ORF, deletion of 121 aa	8	22
NT 300-996	Exon3-7	Frameshift, Truncation	9	23
NT 541-659	Exon 4	Frameshift, Truncation	10	24
NT 659-744	Exon 5	Frameshift, Truncation	11	25
NT 660-996	Exon 5-7	Frameshift, Truncation	12	26
NT 996-1208	Exon 8-9	Frameshift, Truncation	13	27
NT: 229-230 (aa 34)	deletion AG	Gln→Stop at aa 38, nonsense	14	28
NT: 282 (aa 52)	deletion A	Asn→Stop at aa 54, nonsense	15	29
NT: 350-351 (aa 74)	deletion AG	Arg→Stop at aa 78, nonsense	16	30
NT: 136-299	Exon 2	Frameshift, Truncation	17	31

aa = amino acid

NT = nucleotide

Table 2: Replaced amino acids in mPark2 cDNA (SEQ ID NO:1)

Position in SEQ ID NO:1	Replacement (DNA)	Replacement (protein)	SEQ ID NO (DNA seq)	SEQ ID NO (prot seq)
NT: 608	G→T,	Lys→Asn (aa 161)	18	32
NT: 1369	C→A,	Thr→Asn (aa 415)	19	33
NT: 1483	G→A,	Trp→Stop (aa 453)	20	34

aa = amino acid

NT = nucleotide

Once the construct has been prepared and manipulated, the DNA is isolated from the procaryotic host according to any method known in the art. Before the DNA construct is introduced into the target cells for homologous recombination undesired sequences may be removed from the vector, e.g. the undesired bacterial sequences. As target cells an embryonic stem (ES) cell line may be used. As already indicated above for the expression system, any convenient technique for introducing the DNA into the target cells may be employed. After transformation of the target cells, many target cells are selected by means of positive and/or negative markers, as previously indicated, neomycin resistance and Acyclovir or Gancyclovir resistance. Those cells which show the desired phenotype may then be further analyzed by restriction analysis, electrophoresis, Southern analysis, polymerase chain reaction or the like. By identifying fragments which show the presence of the lesion(s) at the target gene site, one can identify cells in which homologous recombination has occurred to inactivate the target gene.

For embryonic stem cells, after mutation, the cells may be plated onto a feeder layer in an appropriate medium, e.g., fetal bovine serum enhanced DMEM. Cells containing the construct may be detected by employing a selective medium and after sufficient time for colonies to grow, colonies

may be picked and analyzed for the occurrence of homologous recombination. As described previously, the polymerase chain reaction may be used, with primers within and without the construct sequence but at the target locus. Those colonies which show homologous recombination may then be used for embryo manipulating by blastocyst injection. Blastocysts may be obtained from 4 to 6 week old superovulated females by flushing the uterus 3.5 days after ovulation. The embryonic stem cells may then be trypsinized and the modified cells added to a droplet containing the blastocysts. At least one, usually at least about 10, and up to about 15 of the modified embryonic stem cells may be injected into the blastocoel of the blastocyst. After injection, at least one and not more than about 15 of the blastocysts are returned to each uterine horn of pseudopregnant females. Alternatively, any of the common techniques, i.g. microinjection of the mutated gene, or a fragment thereof, into a one-cell embryo followed by incubation in a foster mother can be used.

The pups will usually be born 16-18 days after introduction of the blastocysts into foster mothers. Chimeric animals will be mated with wild type (wt) mice to create heterozygote transgenics.

With these methods it is possible to obtain transgenic non-human animals, whose one or both allels of a gene encoding a parkin gene are mutated in a way, that a parkin protein with modified, preferred less activity or no active parkin protein is expressed.

"Mutated" means in this content replacements, insertions or deletions of nucleotides or polynucleotide sequences.

In consequence of the mutated parkin gene these animals produce a mutated or truncated parkin protein or no parkin protein. Preferred - if a parkin protein is expressed - the parkin protein expressed by the transgenic animal contains any of the mutations or deletions shown in table 1 and 2,

represented by any of the proteins with an amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ, ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34 or naturally occurring or artificially introduced mutants with a homologous protein sequence or fragments thereof, particularly preferred a parkin protein with a sequence according to SEQ ID NO:21 is expressed.

The expression of one of these proteins or no parkin protein in the transgenic non-human animals causes these animals to display features of a neurodegenerative disease. These features can be manifested in developing physiological, biochemical or molecular biological modifications in e.g. cells, tissues, organs or neuronal structures.

In accordance with standard protocols, cultured eukaryotic cells, either primary cultures or immortalised cell lines, may be transfected, either transiently or stably, with a mutant or fragmented mPark2 allele so that the cultured eukaryotic cell expresses a mutant parkin2 polypeptide.

The present application further refers to cells, typically mammalian cells and preferably mammalian cells of the neural, glial, or astrocytic lineage, that have been transformed or transfected with any DNA sequence according to the present invention, as well as to any cells which have been derived from a transgenic non-human animal, whereby the cells express any of the mutated parkin2 proteins isoforms according to the present invention, preferred any of the isoforms shown in table 1 or 2 or fragments thereof, or they contain a parkin sequence which is mutated in a way that they don't express a parkin protein. The cells derived from the transgenic animals may be cultured as cell-lines or as primary cultures.

Once established, all such cell lines can be grown continuously in culture and may be used for a variety of in vitro experiments to study parkin expression and processing.

The present invention further refers to a method of producing transgenic non-human animals and transformed cells that contain any polynucleotide sequence encoding any mutant mouse parkin2 protein isoform according to the present invention, preferably such as shown in table 1 or 2 or naturally occurring or artificially introduced mutants or fragments thereof.

Preferred the above described polynucleotide sequences, the proteins and amino acid sequences as well as the transgenic animal models and cell lines may be used for any method for analysing the symptoms of neurodegenerative diseases.

Such neurodegenerative diseases encompass among others Parkinson's disease, Alzheimer's disease, Huntigton's disease, amyotrophic lateral sclerosis, Multisystem atrophy, Wilson's disease, Pick's disease, Prion disease, or second causes inducing Parkinson's syndromes like toxins (e.g. Mn, Fe, 6-hydroxydopamine, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), CO), drugs, brain tumors, head trauma, stroke, vascular irregularities, or metabolic irregularities.

Enclosed to these methods are methods outside of a living body, which are methods of molecular biology like PCR, Southern and Northern blot analysis, construction of DNA or RNA probes, as well as Western blot analysis, preparation of epitopes from the protein or amino acid sequences mentioned in this application, production of monoclonal and polyclonal antibodies. These methods may be used for screening of samples, preferred of biological fluids for either the expression of parkin protein as a method for detecting the presence of the protein, or in a nucleic acid

sample or another sample removed from a subject, the presence of the gene for Parkinson's disease comprising identifying a genetic alteration in a gene sequence coding for parkin. Further enclosed are pathobiochemical, immunobiological and neurological as well as histochemical methods carried out after sacrificing the animal for considering the effects of neurodegenerative diseases, particularly Parkinson's disease to the living body. Further methods for locating the presence of genetic alterations associated with Parkinson's disease are provided. These methods may be used outside of a living body to predict the development of the disease prior to onset or for genetic screening.

However, particularly preferred is a method of testing the efficacy of a treatment for a neurodegenerative disease associated with a less active or non-active parkin protein, comprising subjecting any of the created transgenic animals as a model to a putative treatment and determining the efficacy of said treatment.

These testing methods preferably comprise administering an active substance, whose effect can be determined by any of the above described methods, to a transgenic animal according to the present invention.

By the use of the transgenic animals described in the present application it is possible the first time to test in a model system whether an active substance is useful for treating a condition associated with non-active parkin protein and determining a level of the active substance, which causes an effect in treating the disease.

Treatments may carried out as single dose applications, but it is preferred to use the transgenic animals in long-time experiments with multiple dose applications.

The transgenic animals of the present application may be particularly used as model systems for screening for drugs and evaluating drug effectiveness. Additionally, such model systems provide a tool for defining the underlying biochemistry of neurodegenerative diseases, which thereby provides a basis for rational drug design. The models may be used further for studies of behaviour, physiological and molecular biological examinations, pharmacological and toxicological studies and several other applications.

Having detected the genetic mutation in the gene sequence coding for parkin protein in an individual not yet showing overt signs of Parkinson's disease, using any of the methods of the present invention, it may be possible to employ gene therapy, in the form of gene implants, to prevent the development of the disease.

Additional embodiments directed to modulation of the production of variant parkin proteins include methods that employ specific antisense polynucleotides complementary to all or part of a variant parkin sequence according to any of the sequences mentioned in this application, or for some embodiments a wild-type parkin sequence. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridisation to the relevant target sequence is retained as a property of the polynucleotide. Thus, an antisense polynucleotide must preferentially bind to a variant parkin sequence as compared to a wild-type parkin. It is mostly preferred that the antisense polynucleotide reflects the exact nucleotide sequence of the variant allele (or wild-type allele where desired) and not a degenerate sequence.

Complementary antisense polynucleotides include soluble antisense RNA or DNA oligonucleotides which can hybridise specifically to a variant parkin mRNA species and prevent transcription of the mRNA species and/or

translation of the encoded polypeptide (Ching et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:10006; Broder et al. (1990) Ann. Int. Med. 113:604; Loreau et al. (1990) FEBS Letters 274:53-56); Holcenberg et al. WO91/11535; U.S. Pat. No. 7,530,165 ("New human CRIPTO gene"--publicly available through Derwent Publications Ltd., Rochdale House, 128 Theobalds Road, London, UK); WO91/09865; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). The antisense polynucleotides therefore inhibit production of the variant parkin polypeptides.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell or animal, such as a transgenic neural, glial, or astrocytic cell, preferably where the expression cassette contains a sequence that promotes cell-type specific expression (Wirak et al. loc. cit.). Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in the culture medium in vitro or in the circulatory system or interstitial fluid in vivo. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species. In some embodiments the antisense polynucleotides comprise methylphosphonate moieties. For general methods relating to antisense polynucleotides, see Antisense RNA and DNA, (1988), D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Legends to the figures:

Figure 1 shows the alignment of the deduced amino acid sequences of the human and mouse Parkin2 protein (SEQ ID NO: 4).
Underlinded are the conserved ubiquitin like (at the N-terminus) and Ring finger like (at the C-terminus) regions of both proteins.

Figure 2 shows the alignment of the nucleotide sequences of the human and mouse parkin 2 gene. Bold lines represent the exon boundaries identified for the human and mouse sequence.

Figure 3 represents a flow chart of the cloning procedure of the mouse parkin2 gene - exon3 knock-out construct.

Abbreviations:

a) Restriction endonucleases:

N = NotI, E= Eco RI, B= BamHI, H= HindIII, X= XbaI.

b) Modifications: ()= T4 DNA polymerase treatment in order to remove a restiction site in the resulting plasmid.

c)  = pBluescript KSII (Stratagene) vector sequence

 = λ-Fix vector sequence

d) HSV-tk = herpes simples promotor and thymidine kinase gene

e) kb = kilobases

The following examples are provided for illustration and are not intended to limit the invention to the specific example provided.

Example 1

Isolation of mouse Parkin2 cDNA clones:

Arrayed mouse brain and mouse kidney cDNA libraries (Biofrontera Pharmaceuticals/ Bio Systems) were screened by PCR under standard conditions using the primers Ex2s:*tcaggttcaactccagctatggc* and Ex2as: *tgcctgcgaaaatcacacgcagc* . The cycle conditions were the following: 3 min. 95°C, (30sec. 95°C, 30sec. 56°C, 1min. 72°C) x 35 cycles.

Single colonies containing the mPark2 genes were verified by colony hybridisation according to the protocol described by Maniatis *et al.* 1989 (see above).

Construction of the Del Exon3 parkin gene (according to SEQ ID NO: 7)

All the further described cloning steps are shown in Figure 3. A genomic lambda ZIP clone (genomic mouse λ-Fix library, Stratagene) containing the exon 3 of the parkin gene was isolated by PCR using exon3 specific primer of the mPark2 gene. A 3.1 kb BamHI/HindIII fragment of the lambda ZIP clone containing genomic DNA 3´ end to the exon3 of mPark2 was cloned into the cloning vector pBluescript KS (Stratagene) to obtain the plasmid pmPark2-BH. Secondly, a 5 kb HindIII/EcoRI genomic DNA fragment was inserted into the HindIII site of the pmPark2-BH-clone. The EcoRI and HindIII sites were destroyed by T4 DNA polymerase treatment. As result the plasmid pmPark2-BE- with a 8.1 kb long genomic region to the 3´-end of the exon 3 could be obtained.

A 2.0 kb XbaI/XhoI (the XbaI restriction site is located within the multiple cloning sequence (mcs) of Lambda Fix) genomic DNA fragment containing the genomic region 5' to the exon3 was cloned into the EcoRI site of the pNeoloxp-vector (Giese et al. Science, 1998, 279:870-3) after generation of blunt ends by T4 DNA polymerase treatment. The BamHI-site (5´-to the EcoRI-site) of this vector was used subsequently for the insertion of the 2.5 kb HSV-tk-marker gene. Again T4 DNA polymerase was used to generate blunt ends before ligation in order to eliminate the used cloning site. The resulting vector was digested with the restriction enzymes NotI and XhoI to obtain a 6.5 kb fragment containing the HSV-tk, the 2kb XhoI/XbaI genomic region to 5´-end of exon3, and the neo-marker. The vector pmPark2-BE was digested with XhoI to linearise the plasmid. Both the isolated 6.5 kb fragment as well as the linear vector were

treated with T4 DNA polymerase prior to ligation to eliminate the used restriction sites.

This plasmid pmPark2del-ex3 was linearised with the restriction enzyme NotI prior to transfection into ES cells.

Example 2

Transfection of ES cells:

Isolation and Freezing of the ES cells:

14 days old embryos were isolated, head and organs were removed from embryos, the remaining tissue was minced, and washed with 1x PBS. 1x trypsin (0,5g/l) / EDTA (0,2g/l) was used for dissolve the tissue by incubating them at 37°C for 5 min. The reaction was stopped by adding 1 vol. EF medium (Embryonic Feeder medium: 1x DMEM, 10% FCS Serum, 2mM Glutamine, all obtained from LIFE Technologies), and cells were dissolved by pipetting several time up and down. The supernatant was centrifuged with 1000 rpm for 5 min. The fibroblasts from one embryo were seeded into a 175 cm^2 flask with 30 ml medium. The medium was changed after 24 h. When the fibroblasts form a confluent monolayer they were splitted 1:3, and thereafter they were frozen when the cells are confluent again. Cells from 175 cm^2 flask were frozen into one tube. Therefore first empty tubes are place on ice, freezing medium is added (EF medium + 20% DMSO (Dimethylsulfoxid)), cells with 0.5 ml EF medium are added, mixed, putted in a styrofoam box, which is cooled down in a -80°C freezer, and the next day the tubes are transferred into liquid N_2(l) tank.

Sub-culturing, inactivation and feeder layer:

The fibroblasts can be cultured on gelatine-coated plastic ware. The cells were splitted carefully 1:3 after 3 days. When feeder layer are needed for ES cell culturing, the fibroblasts should be division-inactivated by

mitomycin C. 2 mg mitomycin C are dissolved in 10 ml PBS, which can be stored at -20°C. This stock solution is diluted 1:20 with EF medium for inactivation; the nearly confluent fibroblasts in a 175 cm^2 flask are incubated in 20-30 ml of medium with mitomycin C for 2 h at 37°C. Mitomycin C is then removed by 2x washing with PBS, and the inactivated fibroblasts are recovered in EF medium for 24 h before they are frozen or used for ES cell culturing after a few days. The cells are stored 37°C until they are used (maximally 10 days;) or they are frozen. For feeder layer, plate cells onto the same area; here the plastic ware has to be coated by gelatine.

Sub-culturing the ES-cells:

The ES cells were kept for 2-4 passages in culture. The medium is ES medium (1x DMEM, 15% FCS Serum, 2mM Glutamine, 1x nonessential amino acids, 7μl ß Mercaptoethanol, with supplement containing LIF (Leukemia Inhibitory Factor, $2.5x10^5$ to 10^6 U/l),all obtained from LIFE Technologies), and the cells are splitted 1:6 every second day. Cells were refeeded 2 h before passaging.

Stable Transfection of ES Cells

After digestion of the gene targeting construct the DNA is extracted with phenol/$CHCl_3$ (24/23) and precipitated with EtOH (wash 2x with 75% EtOH); the rest of EtOH is removed carefully and air dryed for approx. 15 min under steril conditions (laminar flow). The DNA is suspended in H_2O (final conc.: 3 mg/ml). $5x10^7$ cells of a monolayer are treated with 1x trypsin to detach them from the ground of the flask, suspended in 0.8 ml medium and electroporated with DNA (30 μg linear DNA, 800 V, 3 μF, BioRad Gene Pulser). After 20 min at 4°C, cells are diluted with 9.5 ml medium and are plated onto dishes (9 cm diameter). 24 h after electroporation G418 (150-175 mg /ml) is added to start selection. The medium is changed every day; after 7-9 days of selection colonies can be picked.

Picking colonies and culturing of picked colonies:

24 colonies were picked with Eppendorf tips under an inverted microscope. The colonies were transferred into the wells of a 96-well plate (round bottom), 30 µl 1xtrypsin/EDTA are added, and the plates are incubated 10 min at 37°C. Thereafter 100 µl ES-medium are added and the cells are suspended by pipetting up and down 12x with a multichannel pipette. The trypsinized cells are solitarily plated into a 24-well plate. The medium is exchanged every 24 h. 3-4 days after picking the cells are detached from the ground of the plates. Therefore the medium is removed, 60 µl 1xtrypsin/EDTA are added and the plates are incubated for 7 min at 37°C. The treatment is stopped by adding 200 µl medium and the cells are resuspended. 200 µl of the cell suspension is added to 200 ml medium with 20 % DMSO and the cells are frozen as described above.

Example 3

DNA isolation and southern blot analysis for control and identification of picked colonies:

To characterize the clones, picked in example 2, DNA is isolated from the cells and examined. Therefore 500 µl medium are added into any well of a picked colony which should still contain 60 µl cell suspension (see example 2). The cells are cultured continuously 3-4 days until confluent for DNA isolation. 500 µl lysis buffer (12 ml 1 M Tris-HCl (pH 8.3); 1.2 ml 0.5 M EDTA; 2.4 ml 10 % SDS; 4.8 ml 5 M NaCl; 1.2 ml 10 mg/ml proteinase K; 98.4 ml H_2O) is added, and it is incubated over night at 55°C. DNA is precipitated by adding 1 vol. 2-propanol and at least 15 min shaking at RT, and transferred with an Eppendorf tip into a 1.5 ml tube with 1 ml 70% EtOH. The tube is centrifuged for 10 min at RT to spin down the DNA. EtOH is removed and pellets are air dried for least one hour. DNA is dissolved afterward in 100 µl TE for over night at 55°C.

Southern blot analysis:

1/3 of the isolated DNA was used for one digestion. The digestion was carried out for over night at 37°C. Loading buffer was added, and DNAs are separated in an agarose gel for least 6 hours. The gels were incubated in 0.2 N HCl for 15 min at room temperature; after 15 min HCl solution was replaced by 0.4 N NaOH and the gel was incubated therein for 15 min at RT. The DNA was transferred onto nylon membranes (Amersham) over night using 0.4 N NaOH as transfer buffer using a vacuum blot machine (Stratagene). The membranes were neutralized in 2x SSC for 1 min, and air dried for least one hour. After UV-Crosslinking the DNA onto the membrane hybridisation with DNA probes (probes are shown in figure 3) was carried out under standard conditions (QuickHyb from Clontech, 65°C, wash twice with 2x SSC, 0.1 % SDS at 65°C).

Production of transgenic animals with mutant parkin allele:

10-15 recombinant ES cells are injected into blastocyts. The blastocyts are implanted in pseudopregnant mice. The chimeric spring offs are crossed with wild type mice to obtain heterocygotic recombinant F1 mice. These mice are analysed by southern blot analysis as described above. Transgenic mice are crossed with each other to obtain mice with both alleles modified (homozygote animals).

Descendants of the transgenic animals may be used for breeding with mice strains representing the same or any other genotype, preferred mice strains showing neurological abnormalities, more preferred with strains showing neurodegenerative abnormalities. These other mouse strains may be selected from wild type mice, mice containing knock-ins or knock-outs, mice containing mutants of genes or mice which overexpress any gene product. The most preferred partners for breeding are mice which represent a model for Alzheimer's disease, Huntigton' disease, amyotrophic lateral sclerosis, Multisystem atrophy, Wilson's disease, Pick's disease or Prion disease.

Use of Transgenic Mice:

The animal can be used to test potential therapeutic agents. The test group of mice is treated with the test compound administered in an appropriate fashion for a set period. At the conclusion of the test period, the animals are assessed behaviourally, biochemically, and histologically for any possible effects of the test compound. The exact protocol depends on the anticipated mechanism of action of the test compound. Compounds that may have utility in treating Parkinson's disease can be identified using this approach.

Such analysis can be carried out in the animal ,in primary tissue cultures of the expressing cells or in immortalised cells derived from those animals.

Mice expressing the truncated parkin2 protein gene or variants of the described one can be used for testing the development of Parkinson's disease during ageing of the animals. Beside the enhanced progression of cell death in substantia nigra area, increased sensitivity to selective neurotoxins like MPTP or 6-hydroxydopamine and enhanced response to dopaminergic precursors like L-dopa may be examined.

Claims

1. A polynucleotide sequence encoding a mouse parkin2 protein, containing naturally occurring or artificially introduced mutations or deletions, which cause Parkinson's disease in a human if they occur in the according human sequence.

2. The sequence of claim 1, wherein the sequence is genomic DNA, coding for a full-length parkin gene or fragments thereof, cDNA of a full length parkin gene or fragments thereof, or RNA of a full length parkin gene or fragments thereof.

3. The sequence of claim 1 or 2, wherein the sequence is selected from the group, consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7 SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or naturally occurring or artificially introduced mutants or fragments thereof.

4. A vector, containing any sequence according to any of claims 1 to 3.

5. A prokaryotic or eukaryotic cell, containing a vector according to claim 4.

6. The cell of claim 5, characterised in that the cell is selected from bacterial or yeast cells, insect cells or mammalian cells as primary cells or immortalised cell lines.

7. A parkin mouse protein with an amino acid sequence of SEQ ID NO:5, SEQ ID NO:6 SEQ, ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34 or

naturally occurring or artificially introduced mutants with a homologous protein sequence or fragments thereof.

8. A transgenic non-human animal, whose one or both alleles of a gene encoding a parkin gene are mutated or truncated in a way, that a protein with modified, preferred less activity or no active protein is expressed.

9. The transgenic animal of claim 8, wherein the parkin gene has any mutation or deletion which are known to cause Parkinson's disease in a human if they occur in the according human sequence.

10. The transgenic non-human animal of claim 8 or 9, carrying a mutation or deletion in one or both alleles of a gene encoding a parkin protein, such that expression of said parkin gene produces a mutated or truncated protein or no protein, which causes said animal to display any physiological, biochemical or molecular biological features of a neurodegenerative disease.

11. The transgenic non-human animal of claim 10, carrying a deletion in one or both alleles of any of the exons of the gene encoding the parkin protein.

12. The transgenic non-human animal of any of claims 8 to 11, carrying a DNA sequence according to any of the sequences SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7 SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19 or SEQ ID NO:20.

13. A mammalian cell-line transformed or transfected with any sequence according to any of claims 1 to 3 or a vector according to claim 4 or

cell lines or primary cultures derived from the transgenic animal of any of claims 8 to 12.

14. A method of producing a transgenic animal according to any of claims 8 to 12 or a cell line according to claim 13.

15. Use of the transgenic non-human animal according to any of claims 8 to 12 or a cell line according to claim 13 as a model for neurodegenerative diseases.

16. A method for analyzing the symptoms of neurodegenerative diseases, either outside of a living body using any of the polynucleotide sequences of any of claims 1 to 4, any of the protein sequences of claim 7, or using any model according to claim 15.

17. A method for testing the efficacy of a treatment for a neurodegenerative disease associated with a less active or non-active parkin protein, comprising subjecting any model of claim 15 to a putative treatment and determining the efficacy of said treatment.

18. The method according to claim 16 or 17, wherein said neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, Huntigton's disease, amyotrophic lateral sclerosis, Multisystem atrophy, Wilson's disease, Pick's disease, Prion disease, or second causes inducing Parkinson's syndromes like toxins, drugs, brain tumors, head trauma, stroke, vascular irregularities, or metabolic irregularities.

19. The method of any of claims 17 to 19, wherein said treatment comprises administering an active substance to the model.

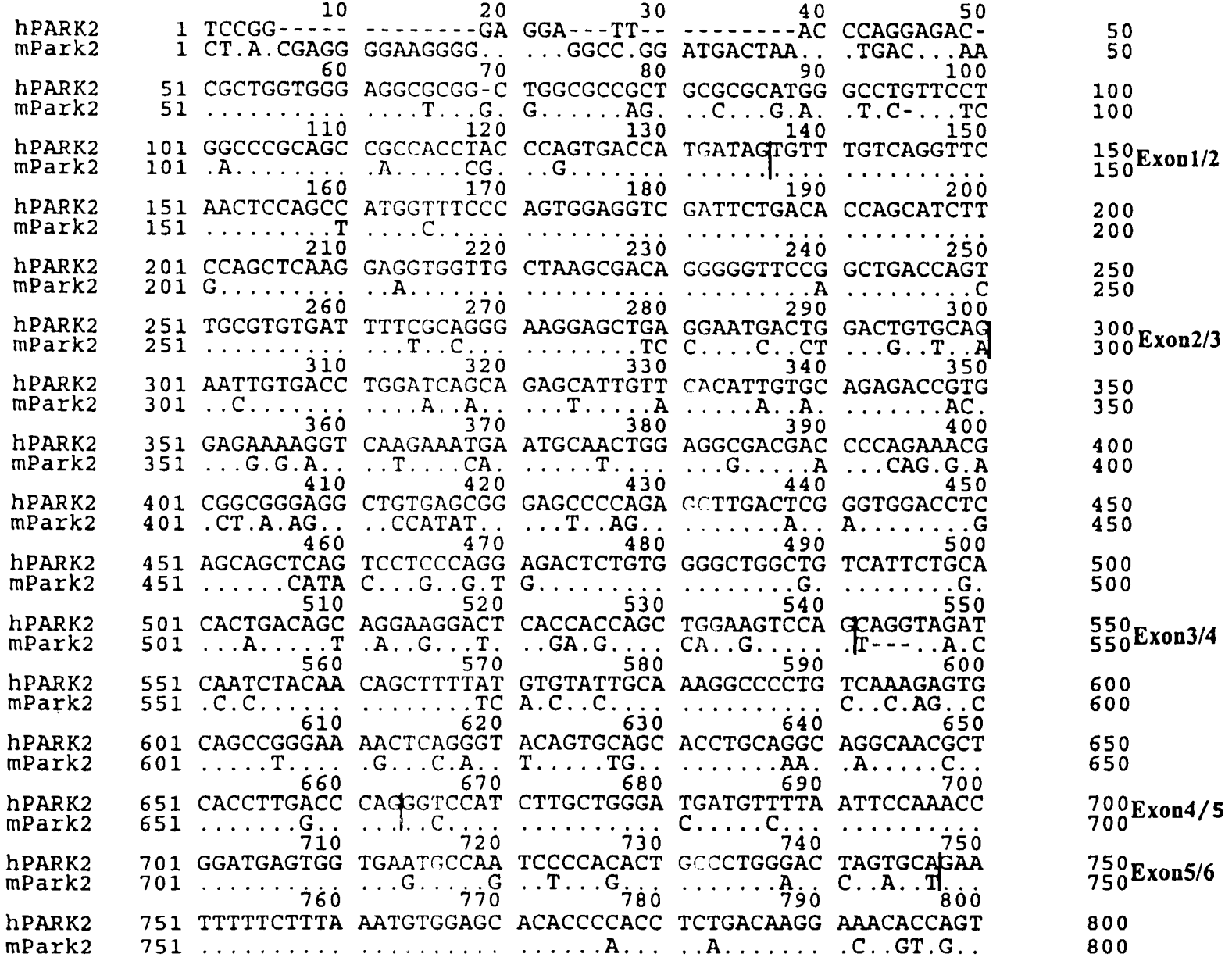
20. Use of any model according to claim 15 for testing whether an active substance is useful for treating a condition associated with non-active parkin protein comprising administering said active substance to the transgenic animal of any of claims 8 to 12 or a cell-line of claim 13, and determining a level of the active substance, which causes an effect in treating the disease.

21. Use of the animal according to any of claims 8 to 12 as a model for examination of behaviour during the development of a neurodegenerative disease, or any model according to claim 15 for examination of pathobiochemical, immunobiological, neurological as well as histochemical effects of neurodegenerative diseases, physiological and molecular biological correlation of the disease, for studies of drug effects and for determination of effective drug doses and toxicity.

22. Descendant of the transgenic animal according to any of claims 8 to 12, obtained by breeding with the same or any other genotype.

```
                        10        20        30        40        50
hPARK2      1 MIVFVRFNSS HGFPVEVDSD TSIFQLKEVV AKRQGVPADQ LRVIFAGKEL 50
mPARK2      1 .........Y..........L.......................... ..... 50

                    60        70        80        90       100
hPARK2     51 RNDWTVQNCD LDQQSIVHIV QRPWRKGQEM NATGGDDPRN AAGGCEREPQ 100
mPARK2     51 P.HL.....E..........R.RSH.T ..S...E.QS TSE.SIW.SR 100

                   110       120       130       140       150
hPARK2    101 SLTRVDLSSS VLPGDSVGLA VILHTDSRKD SPPAGSPAGR SIYNSFYVYC 150
mPARK2    101 .........H T..V..... ...D...KR. .EA.RG.V-K PT....FI.. 150

                   160       170       180       190       200
hPARK2    151 KGPCQRVQPG KLRVQCSTCR QATLTLTQGP SCWDDVLIPN RMSGECQSPH 200
mPARK2    151 ....HK.... ......G..K .....A... .......... .........D 200

                   210       220       230       240       250
hPARK2    201 CPGTSAEFFF KCGAHPTSDK ETPVALHLIA TNSRNITCIT CTDVRSPVLV 250
mPARK2    201 ....R..... .......... D.S...N..T S.R.S.P..A .......... 250

                   260       270       280       290       300
hPARK2    251 FQCNSRHVIC LDCFHLYCVT RLNDRQFVHD PQLGYSLPCV AGCPNSLIKE 300
mPARK2    251 ....H..... .......... .......... A.......... .......... 300

                   310       320       330       340       350
hPARK2    301 LHHFRILGEE QYNRYQQYGA EECVLQMGGV LCPRPGCGAG LLPEPDQRKV 350
mPARK2    301 .......... ..T....... .......... .......... ....QG.... 350

                   360       370       380       390       400
hPARK2    351 TCEGGNGLGC GFAFCRECKE AYHEGECSAV FEASGTTTQA YRVDERAAEQ 400
mPARK2    351 .......... ..V...D... .....D.DSL L.P..A.S.. ....K..... 400

                   410       420       430       440       450
hPARK2    401 ARWEAASKET IKKTTKPCPR CHVPVEKNGG CMHMKCPQPQ CRLEWCWNCG 450
mPARK2    401 ....E..... ......N..I.......... ..........K......... 450

                   460       470       480       490       500
hPARK2    451 CEWNRVCMGD HWFDV*.... .......... .......... .......... 500
mPARK2    451 .....A.....*.... .......... .......... .......... 500
```

Fig. 1


10 20 30 40 50
hPARK2 1 TCCGG----- --------GA GGA---TT-- --------AC CCAGGAGAC- 50
mPark2 1 CT.A.CGAGG GGAAGGGG.. ...GGCC.GG ATGACTAA.. .TGAC...AA 50
60 70 80 90 100
hPARK2 51 CGCTGGTGGG AGGCGCGG-C TGGCGCCGCT GCGCGCATGG GCCTGTTCCT 100
mPark2 51 ....T...G. G......AG. ..C...G.A. .T.C-...TC 100
110 120 130 140 150
hPARK2 101 GGCCCGCAGC CGCCACCTAC CCAGTGACCA TGATAGTGTT TGTCAGGTTC 150
mPark2 101 .A..... .A.....CG. ..G..... 150
Exon1/2
160 170 180 190 200
hPARK2 151 AACTCCAGCC ATGGTTTCCC AGTGGAGGTC GATTCTGACA CCAGCATCTT 200
mPark2 151 .....T ....C..... 200
210 220 230 240 250
hPARK2 201 CCAGCTCAAG GAGGTGGTTG CTAAGCGACA GGGGGTTCCG GCTGACCAGT 250
mPark2 201 G..... ..A..... ....A .....C 250
260 270 280 290 300
hPARK2 251 TGCGTGTGAT TTTCGCAGGG AAGGAGCTGA GGAATGACTG GACTGTGCAG 300
mPark2 251 ....T..C... .....TC C....C..CT ...G..T..A 300
Exon2/3
310 320 330 340 350
hPARK2 301 AATTGTGACC TGGATCAGCA GAGCATTGTT CACATTGTGC AGAGACCGTG 350
mPark2 301 ..C..... ....A..A.. ...T.....A .....A..A. .....AC. 350
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hPARK2 351 GAGAAAAGGT CAAGAAATGA ATGCAACTGG AGGCGACGAC CCCAGAAACG 400
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mPark2 651 .....G.. ....C..... ..... C.....C... 700
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Exon5/6
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Fig 2

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mPark2   1051 .......... ..|....CTA. .......... .....G..C. ....A..C.. 1100 Exon8/9
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mPark2   1101 G.....A... ..A..T...C .G........ T......... .....T..A. 1150
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mPark2   1151 ....A..T.. A.AG.G.... .......... .......... ......C... 1200
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mPark2   1351 ..G....... .......G.. .........T .....T.... ..A.C..G.. 1400
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mPark2   1401 .A.T...... ..C|....A. .T........ .........T .......... 1450 Exon11/12
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Fig 2

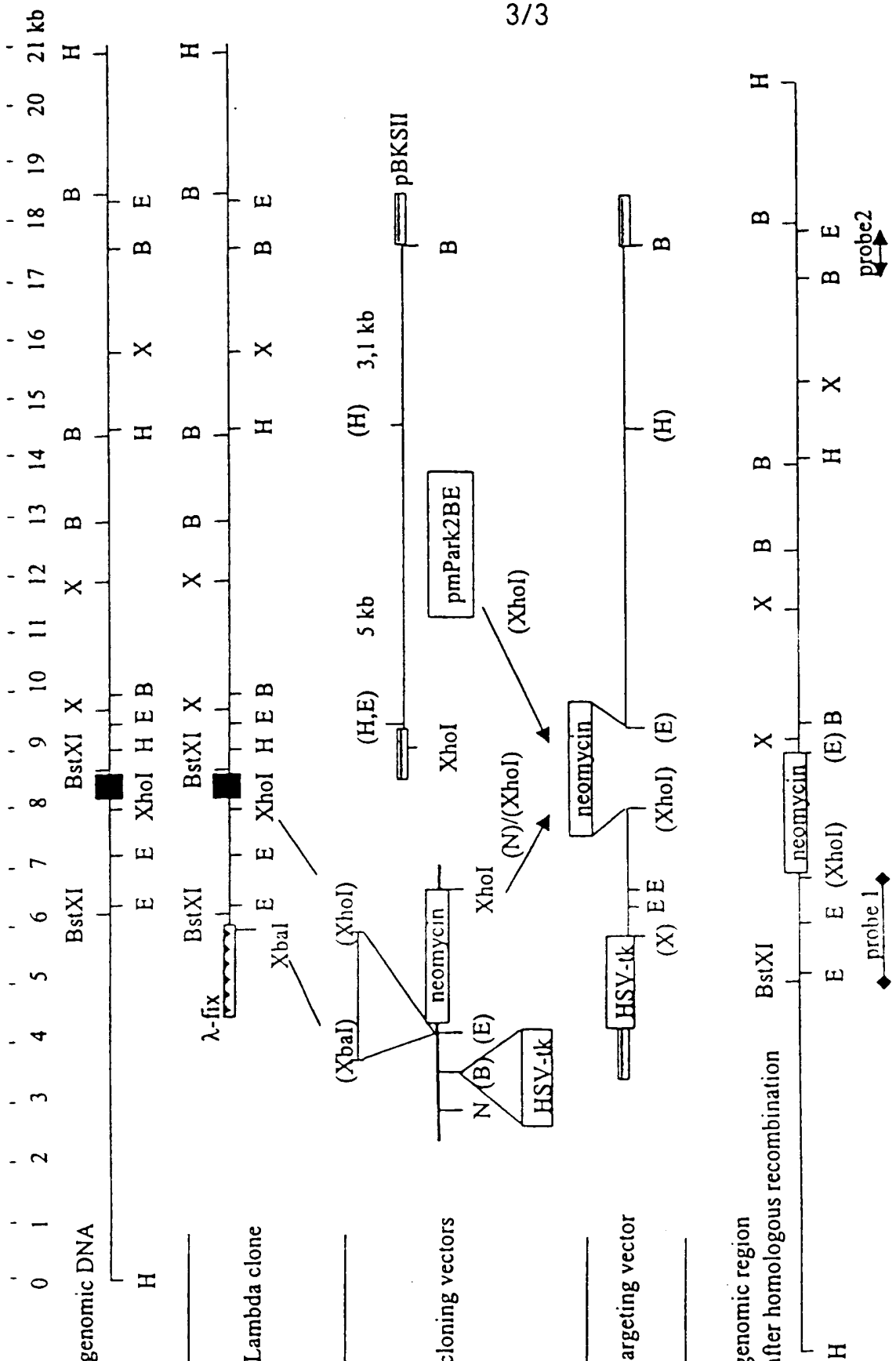
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Fig 3

SEQUENZPROTOKOLL

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Pro Gly Thr Arg Ala Glu Phe Phe Phe Lys Cys Gly Ala His Pro Thr
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275 280 285

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35 40 45

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Glu Leu Pro Asn His Leu Thr Val Gln Asn Cys Asp Leu Glu Gln Gln

50 55 60

Ser Ile Val His Ile Val Gln Arg Pro Arg Arg Arg Ser His Glu Thr
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Glu Leu Pro Asn His Leu Thr Val Gln Asn Cys Asp Leu Glu Gln Gln
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Ser Ile Val His Ile Val Gln Arg Pro Arg Arg Arg Ser His Glu Thr
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35 40 45

Glu Leu Pro Asn His Leu Thr Val Gln Asn Cys Asp Leu Glu Gln Gln
50 55 60

Ser Ile Val His Ile Val Gln Arg Pro Arg Arg Arg Ser His Glu Thr
65 70 75 80

Asn Ala Ser Gly Gly Asp Glu Pro Gln Ser Thr Ser Glu Gly Ser Ile
85 90 95

Trp Glu Ser Arg Ser Leu Thr Arg Val Asp Leu Ser Ser His Thr Leu
100 105 110

Pro Val Asp Ser Val Gly Leu Ala Val Ile Leu Asp Thr Asp Ser Lys
115 120 125

Arg Asp Ser Glu Ala Ala Arg Gly Pro Val Lys Pro Thr Tyr Asn Ser
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Thr Asp Val Arg Ser Pro Val Leu Val Phe Gln Cys Asn His Arg His
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Arg Asp Ser Glu Ala Ala Arg Gly Pro Val Lys Pro Thr Tyr Asn Ser
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Ile Leu Gly Glu Glu Gln Tyr Thr Arg Tyr Gln Gln Tyr Gly Ala Glu
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Ala Thr Ser Gln Ala Tyr Arg Val Asp Lys Arg Ala Ala Glu Gln Ala
385 390 395 400

Arg Trp Glu Glu Ala Ser Lys Glu Thr Ile Lys Lys Thr Asn Lys Pro
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Thr Asp Val Arg Ser Pro Val Leu Val Phe Gln Cys Asn His Arg His
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Asp Arg Gln Phe Val His Asp Ala Gln Leu Gly Tyr Ser Leu Pro Cys
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Gly Cys Glu
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(74) Agent: **STERNAGEL, FLEISCHER, GODEMEYER & PARTNER**; Braunsberger Feld 29, D-51429 Bergisch Gladbach (DE).

(81) Designated States *(national)*: CA, JP, US.

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Published:
— *with international search report*

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27 September 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/16176 A3

(54) Title: TRANSGENIC ANIMAL MODEL FOR NEURODEGENERATIVE DISEASES

(57) Abstract: The present application refers to a mouse parkin2 DNA- and protein sequence, containing mutations or deletions causing Parkinson's disease in a human if occurring in the according human sequence, the construction of a transgenic non-human animal containing such a mutated DNA sequence and therefore expressing no or a less active or non-active parkin protein as well as the use of this transgenic animal as a model for neurodegenerative diseases.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
5807WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 08071	18/08/2000	30/08/1999
Applicant		
BIOFRONTERA PHARMACEUTICALS GMBH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08071

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N5/10 C12N1/21 C12N1/19 C07K14/47
A01K67/027 A61K49/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X L	KESSLER J ET AL: "Investigation of the pathogenic mechanism of parkin mutations" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 25, no. 1-2, 1999, pages 52-Abstract 27.20, XP000884113 29th Annual Meeting of the Society for Neuroscience, Part 1, Miami Beach, Florida, USa, October 23-28, 1999 -& DALIE J E (PROGRAM MANAGER - SOCIETY FOR NEUROSCIENCE): "Publication dates for the 1999 Abstract Volumes" SOCIETY FOR NEUROSCIENCE ABSTRACTS, 16 August 1999 (1999-08-16), XP002157614 --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2001	07/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE EMROD E.M.B.L. Databases; Accession Number: AB019558, 13 July 1999 (1999-07-13) SHIMIZU N ET AL: "Mus musculus mRNA for parkin, complete cds" XP002131476 cited in the application 100% identity in 1644 bp overlap with SeqIdNo.1 abstract	1-22
Y	WO 98 59050 A (JOHNSON WILLIAM G ;LAVEDAN CHRISTIAN (US); NUSSBAUM ROBERT L (US);) 30 December 1998 (1998-12-30) claims 62,63	8-22
Y	GOLDBERG M S ET AL: "STUDIES OF WILD-TYPE AND MUTANT ALPHA-SYNUCLEIN IN TRANSGENIC MICE" ANNUAL MEETING SOCIETY NEUROSCIENCE,XX,XX, vol. 24, no. 1/02, 1998, page 966 XP000884112 the whole document	8-22
Y	KITADA ET AL: "Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 392, no. 6676, 9 April 1998 (1998-04-09), pages 605-608, XP002108469 ISSN: 0028-0836 cited in the application figure 4C & WO 99 40191 A (SHIMIZU NOBUYOSHI ;MIZUNO YOSHIKUNI (JP)) 12 August 1999 (1999-08-12)	1-7
Y	LÜCKING ET AL: "Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 352, no. 9137, 24 October 1998 (1998-10-24), pages 1355-1356, XP002108466 ISSN: 0140-6736 cited in the application the whole document	1-7

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	HATTORI ET AL: "Point Mutations (Thr240Arg and Ala311Stop) in the Parkin Gene" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC. ORLANDO, FL, vol. 249, no. 3, 1998, pages 754-758, XP002108468 ISSN: 0006-291X cited in the application figure 1 ---	1-7
Y	LEROY ET AL: "Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease" HUMAN GENETICS,DE,BERLIN, vol. 103, no. 4, October 1998 (1998-10), pages 424-427, XP002108470 cited in the application figure 1 ---	1-7
Y	ABBAS ET AL: "A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe" HUMAN MOLECULAR GENETICS,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 8, no. 4, April 1999 (1999-04), pages 567-574, XP002108471 ISSN: 0964-6906 cited in the application the whole document ---	1-7
Y	HATTORI N ET AL: "Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals" ANN NEUROL., vol. 44, no. 6, December 1998 (1998-12), pages 935-941, XP000877155 cited in the application table 2 ---	1-7
P,X	WO 00 31253 A (INST NAT SANTE RECH MED ;BOULEY SANDRINE (FR); BRICE ALEXIS (FR);) 2 June 2000 (2000-06-02) claims 39,40 -----	1-22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/08071

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9859050 A	30-12-1998	AU 8163798 A	04-01-1999
WO 0031253 A	02-06-2000	FR 2786199 A	26-05-2000
		FR 2797272 A	09-02-2001
		AU 1276900 A	13-06-2000

PCT REQUEST

5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

0	**For receiving Office use only**	
0-1	International Application No.	PCT/EP 0 0 / 0 8 0 7 1
0-2	International Filing Date	1 8 AUG 2000 (18. 08. 2000)
0-3	Name of receiving Office and "PCT International Application"	EUROPEAN PATENT OFFICE PCT INTERNATIONAL APPLICATION

0-4	**Form - PCT/RO/101 PCT Request**	
0-4-1	Prepared using	PCT-EASY Version 2.91 (updated 10.05.2000)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	5807WO
I	**Title of invention**	TRANSGENIC ANIMAL MODEL FOR NEUROGENERATIVE DISEASES
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	BIOFRONTERA PHARMACEUTICALS GMBH
II-5	Address:	Hemmelrather Weg 201 D-51377 Leverkusen Germany
II-6	State of nationality	DE
II-7	State of residence	DE
III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	LÜBBERT, Hermann
III-1-5	Address:	Höhenstraße 59 D-51381 Leverkusen Germany
III-1-6	State of nationality	DE
III-1-7	State of residence	DE

PCT REQUEST

5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	**Form - PCT/RO/101 PCT Request**	
0-4-1	Prepared using	PCT-EASY Version 2.91 (updated 10.05.2000)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	5807WO
I	**Title of invention**	TRANSGENIC ANIMAL MODEL FOR NEUROGENERATIVE DISEASES
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	BIOFRONTERA PHARMACEUTICALS GMBH
II-5	Address:	Hemmelrather Weg 201 D-51377 Leverkusen Germany
II-6	State of nationality	DE
II-7	State of residence	DE
III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	LÜBBERT, Hermann
III-1-5	Address:	Höhenstraße 59 D-51381 Leverkusen Germany
III-1-6	State of nationality	DE
III-1-7	State of residence	DE

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PCT REQUEST 5807WO

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IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name	STERNAGEL, FLEISCHER, GODEMEYER & PARTNER
IV-1-2	Address:	Braunsberger Feld 29 D-51429 Bergisch Gladbach Germany
IV-1-3	Telephone No.	+49-2204-98560
IV-1-4	Facsimile No.	+49-2204-985625
IV-1-5	e-mail	bgl@polypatent.de
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	CA JP US
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	**Exclusion(s) from precautionary designations**	NONE
VI-1	**Priority claim of earlier regional application**	
VI-1-1	Filing date	30 August 1999 (30.08.1999)
VI-1-2	Number	99116766.9
VI-1-3	Regional Office	EP
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)

PCT REQUEST 5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

VII-2	**Request to use results of earlier search; reference to that search**		
VII-2-1	Date	24 February 2000 (24.02.2000)	
VII-2-2	Number	99 116 766.9	
VII-2-3	Country (or regional Office)	EP	
VIII	**Check list**	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description (excluding sequence listing part)	32	-
VIII-3	Claims	4	-
VIII-4	Abstract	1	5807ep__abstract.txt
VIII-5	Drawings	4	-
VIII-6	Sequence listing part of description	39	-
VIII-7	TOTAL	84	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-15	Nucleotide and/or amino acid sequence listing in computer readable form		separate diskette
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**	None	
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**		
IX-1-1	Name	STERNAGEL, FLEISCHER, GODEMEYER & PARTNER	
IX-1-2	Name of signatory	Dr. Holm Fleischer	
IX-1-3	Capacity	Representative (Association No. 144)	

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	

PCT REQUEST

5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

PCT (ANNEX - FEE CALCULATION SHEET)

5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

(This sheet is not part of and does not count as a sheet of the international application)

0 0-1	**For receiving Office use only** International Application No.			
0-2	Date stamp of the receiving Office			

0-4 0-4-1	**Form - PCT/RO/101 (Annex)** **PCT Fee Calculation Sheet** Prepared using	PCT-EASY Version 2.91 (updated 10.05.2000)		
0-9	**Applicant's or agent's file reference**	5807WO		
2	**Applicant**	BIOFRONTERA PHARMACEUTICALS GMBH, et al.		
12	**Calculation of prescribed fees**	fee amount/multiplier	total amounts (EUR)	
12-1	Transmittal fee T	⇨	102	
12-2	Search fee S	⇨	945	
12-3	International fee Basic fee (first 30 sheets) b1	409		
12-4	Remaining sheets	54		
12-5	Additional amount (X)	9		
12-6	Total additional amount b2	486		
12-7	b1 + b2 = B	895		
12-8	Designation fees Number of designations contained in international application	4		
12-9	Number of designation fees payable (maximum 8)	4		
12-10	Amount of designation fee (X)	88		
12-11	Total designation fees D	352		
12-12	PCT-EASY fee reduction R	-126		
12-13	Total International fee (B+D-R) I	⇨	1.121	
12-14	Fee for priority document Number of priority documents requested	1		
12-15	Fee per document (X)	30		
12-16	Total priority document fee P	⇨	30	
12-17	**TOTAL FEES PAYABLE (T+S+I+P)**	⇨	2.198	
12-19	**Mode of payment**	authorization to charge deposit account		
12-20	**Deposit account instructions** The receiving Office:	European Patent Office (EPO) (RO/EP)		
12-20-1	is hereby authorized to charge the total fees indicated above to my deposit account	✓		
12-20-2	is hereby authorized to charge any deficiency or credit any over-payment in the total fees indicated above to my deposit account	✓		

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PCT-EASY INFORMATION SHEET

(For applicant use only, DO NOT submit this sheet with the international application)

VALIDATION LOG

	States
Green?	More designations could be made. The following States have not been designated: AP:(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW); EA:(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); OA:(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG); AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CH, LI, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW. Please verify.
	Names
Green?	Applicant 1.:Telephone No. missing
Green?	Applicant 1.:Facsimile No. missing
	Contents
Yellow!	The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
	Fees
Green?	Please confirm that fee schedule utilized is the latest available
	Payment
Green?	Please ensure that you have a valid deposit account with the receiving Office selected.

Before submitting the International Application, please carefully verify that:

-the information contained on printed Request form is correct;
-Box IX of the Request form and item 12-22 of the Annex to the Request form have been signed;
-all elements of the international application as indicated in Box VIII of the Request form have been attached; and,
-the diskette containing the PCT-EASY zip file of the International Application has been enclosed and has been clearly labeled "PCT-EASY", with the applicant's or agent's file reference, and the first applicant's name.

ATTENTION

DO NOT modify any indications on the Request form printout. The attached PCT-EASY application has been locked. If an error or an omission is discovered at this time, you must copy the submitted application as a template and make the change or correction in a new application (using the submitted application as a template). You may create such a template by copying the submitted application from the "Stored Forms" folder to the "New PCT Forms" folder. Open the new (.0WO) file created in the "New PCT Forms" folder, correct the errors and proceed with the submission process again.

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PCT (ANNEX - FEE CALCULATION SHEET) 5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

12-20-3	is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account	✓
12-21	Deposit account No.	28 000 797
12-22	Date	17 August 2000 (17.08.2000)
12-23	Name and signature	STERNAGEL, FLEISCHER, GODEMEYER & PARTNER

VALIDATION LOG AND REMARKS

13-2-2	Validation messages States	Green? More designations could be made. The following States have not been designated: AP:(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW); EA:(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); OA:(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG); AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CH, LI, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW. Please verify.
13-2-3	Validation messages Names	Green? Applicant 1.:Telephone No. missing
		Green? Applicant 1.:Facsimile No. missing
13-2-6	Validation messages Contents	Yellow! The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
13-2-7	Validation messages Fees	Green? Please confirm that fee schedule utilized is the latest available
13-2-8	Validation messages Payment	Green? Please ensure that you have a valid deposit account with the receiving Office selected.

PCT

5807WO

Original (for **SUBMISSION**) - printed on 17.08.2000 11:17:30 AM

PCT-EASY INFORMATION SHEET

(For applicant use only, DO NOT submit this sheet with the international application)

VALIDATION LOG

	States
Green?	More designations could be made. The following States have not been designated: AP:(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW); EA:(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); OA:(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG); AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CH, LI, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW. Please verify.
	Names
Green?	Applicant 1.:Telephone No. missing
Green?	Applicant 1.:Facsimile No. missing
	Contents
Yellow!	The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
	Fees
Green?	Please confirm that fee schedule utilized is the latest available
	Payment
Green?	Please ensure that you have a valid deposit account with the receiving Office selected.

Before submitting the International Application, please carefully verify that:

-the information contained on printed Request form is correct;
-Box IX of the Request form and item 12-22 of the Annex to the Request form have been signed;
-all elements of the international application as indicated in Box VIII of the Request form have been attached; and,
-the diskette containing the PCT-EASY zip file of the International Application has been enclosed and has been clearly labeled "PCT-EASY", with the applicant's or agent's file reference, and the first applicant's name.

ATTENTION

DO NOT modify any indications on the Request form printout. The attached PCT-EASY application has been locked. If an error or an omission is discovered at this time, you must copy the submitted application as a template and make the change or correction in a new application (using the submitted application as a template). You may create such a template by copying the submitted application from the "Stored Forms" folder to the "New PCT Forms" folder. Open the new (.0WO) file created in the "New PCT Forms" folder, correct the errors and proceed with the submission process again.

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

PCT

To:
STERNAGEL, FLEISCHER,
GODEMEYER & PARTNER
Braunsberger Feld 29
D-51429 Bergisch Gladbach
ALLEMAGNE

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing *(day/month/year)*	20. 09. 2000
Applicant's or agent's file reference: 5807WO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP 00/08071	18/08/2000	30/08/1999

Applicant
BIOFRONTERA PHARMACEUTICALS GMBH

Title of the invention

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.
2. The applicant is further notified that the record copy of the international application was transmitted to the International Bureau on the above date of mailing.
3. ☐ Other:

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

Name and mailing address of the receiving Office	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	N. MAILLIARD Tel.: (070) 340.28.55 The Hague N. Mailliard

PATENT COOPERATION TREATY

PCT

Sternagel, Fleischer, Godemeyer & Partner

07. März 2001

eingegangen/received

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
5807WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 08071	18/08/2000	30/08/1999

Applicant

BIOFRONTERA PHARMACEUTICALS GMBH et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5 With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08071

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N5/10 C12N1/21 C12N1/19 C07K14/47
A01K67/027 A61K49/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	KESSLER J ET AL: "Investigation of the pathogenic mechanism of parkin mutations" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 25, no. 1-2, 1999, pages 52-Abstract 27.20, XP000884113 29th Annual Meeting of the Society for Neuroscience, Part 1, Miami Beach, Florida, USa, October 23-28, 1999	1-22
L	-& DALIE J E (PROGRAM MANAGER - SOCIETY FOR NEUROSCIENCE): "Publication dates for the 1999 Abstract Volumes" SOCIETY FOR NEUROSCIENCE ABSTRACTS, 16 August 1999 (1999-08-16), XP002157614 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2001	07/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax. (+31-70) 340-3016	Lonnoy, O

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE EMROD E.M.B.L. Databases; Accession Number: AB019558, 13 July 1999 (1999-07-13) SHIMIZU N ET AL: "Mus musculus mRNA for parkin, complete cds" XP002131476 cited in the application 100% identity in 1644 bp overlap with SeqIdNo.1 abstract	1-22
Y	WO 98 59050 A (JOHNSON WILLIAM G ;LAVEDAN CHRISTIAN (US); NUSSBAUM ROBERT L (US);) 30 December 1998 (1998-12-30) claims 62,63	8-22
Y	GOLDBERG M S ET AL: "STUDIES OF WILD-TYPE AND MUTANT ALPHA-SYNUCLEIN IN TRANSGENIC MICE" ANNUAL MEETING SOCIETY NEUROSCIENCE,XX,XX, vol. 24, no. 1/02, 1998, page 966 XP000884112 the whole document	8-22
Y	KITADA ET AL: "Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 392, no. 6676, 9 April 1998 (1998-04-09), pages 605-608, XP002108469 ISSN: 0028-0836 cited in the application figure 4C & WO 99 40191 A (SHIMIZU NOBUYOSHI ;MIZUNO YOSHIKUNI (JP)) 12 August 1999 (1999-08-12)	1-7
Y	LÜCKING ET AL: "Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 352, no. 9137, 24 October 1998 (1998-10-24), pages 1355-1356, XP002108466 ISSN: 0140-6736 cited in the application the whole document	1-7

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	HATTORI ET AL: "Point Mutations (Thr240Arg and Ala311Stop) in the Parkin Gene" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC. ORLANDO, FL, vol. 249, no. 3, 1998, pages 754-758, XP002108468 ISSN: 0006-291X cited in the application figure 1 ---	1-7
Y	LEROY ET AL: "Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease" HUMAN GENETICS,DE,BERLIN, vol. 103, no. 4, October 1998 (1998-10), pages 424-427, XP002108470 cited in the application figure 1 ---	1-7
Y	ABBAS ET AL: "A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe" HUMAN MOLECULAR GENETICS,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 8, no. 4, April 1999 (1999-04), pages 567-574, XP002108471 ISSN: 0964-6906 cited in the application the whole document ---	1-7
Y	HATTORI N ET AL: "Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals" ANN NEUROL., vol. 44, no. 6, December 1998 (1998-12), pages 935-941, XP000877155 cited in the application table 2 ---	1-7
P,X	WO 00 31253 A (INST NAT SANTE RECH MED ;BOULEY SANDRINE (FR); BRICE ALEXIS (FR);) 2 June 2000 (2000-06-02) claims 39,40 -----	1-22

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/08071

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9859050 A	30-12-1998	AU 8163798 A	04-01-1999
WO 0031253 A	02-06-2000	FR 2786199 A	26-05-2000
		FR 2797272 A	09-02-2001
		AU 1276900 A	13-06-2000

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
STERNAGEL, FLEISCHER,
GODEMEYER & PARTNER
Braunsberger Feld 29
D-51429 Bergisch Gladbach
GERMANY

Sternagel, Fleischer, Godemeyer & Partner
07. März 2001
eingegangen/received

	Date of mailing *(day/month/year)* 07/03/2001
Applicant's or agent's file reference 5807WO	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/EP 00/08071	International filing date *(day/month/year)* 18/08/2000
Applicant BIOFRONTERA PHARMACEUTICALS GMBH et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Carla Louro

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet orginally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.